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Characterization of the Dna From Bovine Erythrocytes Infected With Anaplasma Marginale.

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CHARACTERIZATION OF THE DNA FROM BOVINE ERYTHROCYTES INFECTED WITH *ANAPLASMA MARGINALE*

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TO ROBERTA

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ABSTRACT

Recent studies on the DNA of Anaplasma marginale have shown it to be single-stranded. The mole percent adenine, thymine, guanine, and cytosine were determined as 33.8, 17.8, 34.3, and 15.5, respectively. However, by employing more critical means of analysis and characterization its double-stranded nature was established. The DNA is denaturable by formaldehyde. The amount of ethidium bound by the DNA decreases after denaturing by heat. Heated and fast-cooled DNA displayed a slight shift in its absorbance maximum in the presence of 0.04 M Mg^{++}. Native, untreated DNA did not exhibit this phenomenon. A combination of pronase to digest RBC membranes and destroy nucleases and phenol to extract DNA was employed. Equilibrium density centrifugation in CsCl revealed that two DNA species were present whether one used partially-purified organisms or washed infected RBCs. One species possessed a buoyant density of 1.700 $g \cdot cm^{-3}$ and a T_M of 84.9°C, corresponding to normal bovine DNA. The second had buoyant density of 1.710 $g \cdot cm^{-3}$ and a T_M of 92°C and was shown to be present only in A. marginale infected RBCs. Its concentration increased concomitantly with the percentage of infected cells. Electron micrographs of DNA have revealed the presence of circular molecules. Such molecules are thought to originate from A. marginale since bovine DNA has not been shown to be circular. RNA unique to A. marginale has yet to be isolated.

INTRODUCTION

Anaplasmosis of cattle is characterized by acute anemia, enlargement of the spleen, icterus, elevated body temperature, and prostration. The presence of marginal inclusion bodies in erythrocytes is indicative of this disease. The causative agent is termed Anaplasma marginale. Oglesby (1962) estimated that a loss of $35 million annually to the cattle industry in the United States is attributable to anaplasmosis.

Agreement on taxonomy of A. marginale has not been reached (Roby, 1960). This organism has been classified as protozoan parasite (Theiler, 1910; Lotze, 1946; Espana et al., 1959; Cane et al., 1963), a virus (Foote, 1954; Foote et al., 1956), and a rickettsial organism (Philip, 1956; Descaseaux, 1924; Ristic, 1965).

Research on the immunochemistry, pathology, and biochemistry of anaplasmosis has intensified in recent years. The chemical nature of the organism, however, still remains a fertile area for investigation. The marginal body has been shown to contain deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Penha, 1930; Moulton and Christensen, 1955; Gainer, 1961; Gough, 1963). Ellender and Dimopoullos (1967) reported isolating only DNA from purified marginal bodies. The unusual result was that the DNA possessed none of the characteristics of double-stranded, helical DNA.

The work initiated here was to further characterize the DNA extracted from A. marginale obtained from infected erythrocytes.

SELECTED LITERATURE

A. Anaplasmosis

Smith and Kilborne (1893) were among the first to observe marginal bodies in the erythrocytes of cattle suffering from the disease now known as anaplasmosis. They concluded that these bodies were but one part of the life cycle of Piroplasma bigeminum, the causative agent of Texas cattle fever.

By 1910 enough evidence had accumulated for Theiler (1910) to name the disease anaplasmosis and the organism responsible, Anaplasma marginale. The name is descriptive in that the marginal body lacks cytoplasm and is found at the margin of the erythrocyte. The first case of anaplasmosis in the United States was recorded by Meyer (1913), but it was not until 1926 that it was recognized as a separate and distinct disease (Darlington, 1926).

The symptoms of anaplasmosis are reduced milk production, labored respiration, icterus, increased body temperature, disoriented behavior, weakness, and anemia (Carricaburu, 1956a, b). Generally, adult animals contract a more severe case than calves (Christensen, 1956).

The morphology of the marginal body has been studied using different methods of preparation and staining (Dikmans, 1933a, b; Lotze and Yiengst, 1942). It has been shown to be 0.2 μ to 1.0 μ in diameter. Tail-like projections from the marginal body were observed

in Giemsa-stained preparations (Franklin and Redmond, 1958). Espana et al. (1959) observed that the dumbbell-like appearance of Anaplasma bodies may be due to connections between the tail-like projections.

The electron microscope has revealed that the Anaplasma body contained subunits ranging in number from 1-8 (deRobertis and Epstein, 1951; Foote et al., 1958; Ristic, 1960). Such terms as sporoids, elementary bodies, and initial bodies have been applied to these subunits.

Though still obscure the chemical nature of Anaplasma has been the subject of many investigations. Using various staining procedures Moulton and Christensen (1955) reported positive reactions for inorganic ferrous iron, a variety of amino acids, protein, RNA, and DNA. Catalase has been found in Anaplasma (Wallace and Dimopoullos, 1965) as has lactic dehydrogenase (Darre, 1966) and adenosine triphosphatase (Garon, 1966).

Gough (1963) demonstrated that Anaplasma-infected erythrocytes possessed a high concentration of both DNA and RNA. The maximum level of DNA occurred during the peak in marginal body counts, whereas RNA reached a maximum 3 or 4 days later. Recently (Ellender, 1966; Ellender and Dimopoullos, 1967) DNA isolated from purified marginal bodies was reported as having none of the characteristics of a double-stranded, helical molecule.

Glutaraldehyde-fixed infected erythrocytes were shown to contain granules scattered or clumped within the initial body. The granules were partially removed by both DNase and RNase and therefore were thought

to be composed of RNA and DNA (Simpson, 1967). An affinity for uranyl acetate was also displayed, adding further evidence for that conclusion.

B. Nucleic Acids

1. Isolation

The isolation of DNA is an extremely critical step if one wishes to draw any conclusions as to its native state. Extraction is initiated by disrupting the organism or cell employing such techniques as osmotic lysis, enzymes, bacteriophage, surface tension depressants, freezing and thawing, sonication, or high-speed mincing.

Lysis of the organism or cell liberates not only DNA, but RNA, protein, polysaccharide, lipid and inorganic materials. The released DNA is susceptible to degradation by nucleases, the activity of which must be inhibited immediately. Sodium citrate (Peterman and Lamb, 1949), sodium ethylenediamine tetraacetate (EDTA) (Schildkraut et al., 1962), sodium dodecyl sulfate (Kay and Dounce, 1953), Cu^{++} (Zittle, 1945), and nitrogen mustard (Wheeler and Alexander, 1957) have all been used to inhibit the action of these enzymes. Pronase is a proteolytic enzyme of broad specificity which reduces many proteins to amino acids (Thomas et al., 1966). It has been shown that purified Hemophilus DNA or purified T2 DNA survive digestion with pronase without further fragmentation (Berns and Thomas, 1965). This was interpreted to mean that pronase destroys nuclease activity.

Liberated nucleic acid must be freed of protein and lipid. Protein removal is accomplished by using chloroform or carbon tetrachloride

and isoamyl alcohol or octanol (DuBuy et al., 1965; Sevag et al., 1938; Smith and Stokes, 1951), urea (Wyatt and Cohen, 1953), sodium lauryl sulfate (SLS) (2% final concentration) (Marmur, 1961), hot phenol (Massie and Zimm, 1965), or phenol and pronase (Thomas et al., 1966). Lipid from Anaplasma infected bovine cells was removed using an alcohol-ether mixture (Gough, 1963). Whitfeld (1953) used acetone-alcohol, chloroform, and alcohol-ether to remove the lipid from nucleic acid preparations of Plasmodium berghei.

Polysaccharides have been removed from nucleic acid extracts by electrophoresis (Zamenhof et al., 1952). Seibert (1940) used repeated electrophoresis at pH 7.3 to separate polysaccharide and nucleic acids from tubercular protein. Marmur (1961) reported that 2-propanol will selectively precipitate and separate DNA from polysaccharide in a method that has become a classic.

The purified DNA is often precipitated and spooled on a rod (Marmur, 1961). Double-stranded DNA is known to be a relatively rigid thread-like molecule. This rigidity facilitates winding it around a glass rod.

Single-stranded DNA undergoes reversible transitions between extended and compact forms as a function of pH and ionic strength (Studier, 1965). The compact form is stable at high ionic strength if the DNA remains untitrated. Expansion of DNA upon either acid or alkaline titration of the bases cannot be explained by charge effects. Changes in absorbance at 260 nm accompany the transition between the extended and compact forms. These results suggest that base-base interactions are

the important factor in establishing the compact form. It, therefore, seems probable that denatured DNA in high salt concentration at neutral pH would be in the compact form because of intra-strand base-base interaction.

Single-stranded DNA molecules would assume a globular conformation under extraction conditions. Based on these considerations there is no theoretical reason for such DNA molecules to wind on a rod. The procedure designed by Thomas et al. (1966) employs repeated phenolic extractions followed by dialysis which eliminates the need for precipitating the DNA and of winding it on a rod. This method yields high molecular weight preparations.

Removal of RNA is an integral part of isolating DNA, and is most often accomplished by the use of RNase (Mamur, 1961). Hotchkiss (1957) reported that it was difficult to remove the nuclease and that in the presence of DNA, its action was sluggish. The separation can be achieved by electrophoresis (Chargaff and Saidel, 1949; Chargaff and Zamenhof, 1948), calcium salt precipitation (Chargaff and Zamenhof, 1948), and adsorption of RNA on activated charcoal (Zamenhof and Chargaff, 1951). More modern methods include separation in dextran-polyethylene glycol systems (Alberts, 1967) and on methylated-albumin kieselguhr columns (MAK) (Osawa and Sibatani, 1967).

After isolation and purification, the nucleic acid displays increased susceptibility to degradation by the environment (e.g., acid or nucleases) and microorganisms. Lewis (1964) reported that refrigeration

and lyophilization would preserve nucleic acid preparations; however, freezing of DNA in solution produced undesired degradation. Storage for long periods in toluene or under N_2, he noted, produced no detrimental effects.

2. Chemical Analysis

a. Hydrolysis

Hydrolysis of nucleic acids can be accomplished by acid, alkali, or enzymes (Marigold, 1965). The purine-pentose bond is more labile to perchloric or formic acid than the pyrimidine-pentose linkage. Nucleotides and nucleosides are released along with the nitrogenous bases, the relative proportion of each product being dependent upon the length of time of treatment with acid.

Dilute solutions of sodium or potassium hydroxide degrade DNA to a mixture of high molecular weight polynucleotides, RNA being reduced to mononucleotides (Marigold, 1965). Hydrogen peroxide liberates all four bases from DNA by oxidizing the C-1 carbon of deoxyribose to produce deoxyribonic acid (Rhaese and Freese, 1968; Rhaese et al., 1968).

Enzymatic degradation of DNA is accomplished by using DNase. Pancreatic deoxyribonuclease I digests polydeoxyribonucleotides (Hoard and Goud, 1968), and hydrolyzes native DNA in a typically endonucleolytic manner, producing fragments terminated in 5'-monophosphates (Laskowski, 1966). Exonuclease I (Phosphodiesterase) from Escherichia coli catalyzes the hydrolysis of single-stranged polydeoxyribonucleotides. It proceeds in a stepwise manner beginning at the 3'-hydroxyl end of the chain, producing deoxyribonucleoside-5'-monophosphates (Lehman, 1966).

b. Separation and Analysis

Paper chromatography is a method used for the resolution of complex mixtures. One can identify a separated compound by comparing its location with that of a known sample. Eluted experimental and "authentic" spots are quantitated against controls obtained by eluting closely located blank spots of identical dimensions. In spite of these precautions spectra are usually distorted (Laskowski, 1967).

The nucleic acid hydrolysate may be separated on Whatman No. 1 filter paper (Markham and Smith, 1949; Whitfeld, 1953) or Schleicher and Schuell No. 597 (Vischer and Chargaff, 1948; Bendich, 1957). Both of these papers are used because they require no pretreatments (Kream and Chargaff, 1952) and exhibit a high resolving capacity.

The solvent system chosen to separate the nucleic acid components is of primary importance. To separate the nitrogenous bases Wyatt (1951) used 2-propanol-HCl-H_2O(65/16.7/18.3-v/v/v). This solvent allowed the best quantitation of guanine. Other solvent systems used are n-butanol-diethylene glycol-0.1 N HCl (4/1/1 , v/v/v) (Vischer and Chargaff, 1948b), glacial formic acid-n-butanol saturated with water (90/10, v/v) (Markham and Smith, 1949), and 5% Na_2HPO_4-isoamyl alcohol or n-butanol (saturated with 10% aqueous urea) (Carter, 1950).

Detection of the purine and pyrimidines is best achieved with long wave ultraviolet light (UV) (Bendich, 1957). The bases can be eluted by immersing the excised spots in water or 0.1 N HCl for six hours (Heppel, 1967). Quantitation of the eluates is carried out using the molar extinction coefficient for each base.

Use of thin-layer chromatography with anion-exchanger has the following advantages over the older, paper chromatographic methods (Randerath, 1966): (1) complex mixtures of nucleotides can be separated; (2) it is 50-100 times as sensitive as paper chromatography; (3) separation can often be carried out in 5-80 minutes; and (4) experimental conditions (e.g., acidity of solvent) are such as to lessen the danger of nucleotide decomposition. A suitable solvent for separating the nitrogenous bases is methanol-concentrated HCl-H_2O (65:17:18) (Keck and Hagen, 1964).

DeLey (1967) derived a formula for quickly predicting, from spectrophotometric measurements, the mole percent guanine plus cytosine (GC), which is:

$$GC = 168.6\text{-}87.4\ (E_{260}/E_{280})$$

This formula is accurate to within $\pm$ 3% GC, as long as the percent GC is in the range of 40-70%. The extinction ratio is determined using a solution of DNA in 0.1 N acetic acid at pH 3.

3. Thermal Denaturation

The principal effect of elevated temperature on any compound in general, and on DNA in particular, is to supply enough kinetic energy to disrupt the bonds between atoms and molecules. Denaturation (melting) of native, double-stranded DNA may be thought of as consisting of three overlapping steps. The first is the collapse of the hydrogen bonded, double-helical structure followed by the collapse of base-stacking. Finally, the reaction is completed by the dissociation or complete separation of the complementary strands. These steps are reversed by slow cooling.

Denaturation of DNA becomes irreversible when the temperature is abrubtly lowered. Thermally-induced strand separation is a rather slow process requiring about three seconds (Szybalski, 1967). The rate is dependent on the amount of guanine (G) and cytosine (C) present. This reaction is usually followed by heating a DNA solution in a quartz cuvette, while recording the change in absorbance (at 260 nm) as a function of temperature. The resulting curve exhibits a sharp transition over a short range of temperature and the midpoint is defined as the melting temperature, T_M. Knittel *et al*. (1968) described a method using normal probability graphs to determine the T_M from as little as two or three experimentally determined points.

Marmur and Doty (1962) derived an equation for calculating the mole percent guanine plus cytosine (GC) based on the T_M. in degrees centigrade.

$$T_M = 69.3 + 0.41\ (GC)$$

This equation was derived at using a reaction mixture of 20 µg DNA/ml of 1 x SSC (0.15 M NaCl + 0.015 M sodium citrate, pH 7.0 $\pm$ 0.3). They showed that calf thymus DNA reduced by sonic disintegration to a molecular weight of 2.6×10^5 had the same T_M as native DNA with a molecular weight of 8×10^6. The presence of the unusual base hydroxymethylcytosine in DNA (*e.g.* of bacteriophage T2, T4, and T6) does not displace the T_M value predicted by the Marmur-Doty formula.

4. Chemical Reactions

a. Formaldehyde

Haselkorn and Doty (1961) have shown that formaldehyde inactivates virus due to interaction with the amino groups of adenine, cytosine, and guanine. Products of formylation may be Schiff bases of the type R-N = CH_2. Staehelin (1958) and Zamenhof et al. (1953) reported that native DNA does not react with formaldehyde under the same conditions used for reaction with RNA. They concluded that amino groups involved in hydrogen bonds are unreactive toward formaldehyde for a given set of experimental conditions and should reflect quantitatively the fraction of amino groups involved in hydrogen bonds.

Single-stranded DNA or denatured DNA will react with formaldehyde causing an increase in absorbance and a shift in the absorbance maximum (Soehner et al., 1955; Lewin, 1966; Berns and Thomas, 1961). It is thought that the reaction of formaldehyde with the bases prevents the formation of the compact denatured form (Studier, 1965).

A secondary reaction of formaldehyde with DNA includes cross-linking of complementary strands (Zamenhof et al., 1953). The formation of stable methylene bridges between nucleotides is believed to be another reaction, perhaps a primary one (Collins and Guild, 1968).

b. Magnesium Ion

Divalent cations have been shown to exert different effects when they interact with DNA. Ca^{++} and Mg^{++} have both been reported to increase the stability and T_M of DNA whereas Cu^{++} decreased the T_M (Eichhorn, 1962; Eichhorn and Clark, 1965).

It was shown that the combination of either Hg^{++} or Cu^{++} with denatured DNA led to a spectral shift of the maximum absorption to longer wavelengths (Hiai, 1965; Eichhorn et al., 1966). These results were believed to result from the binding of the cations to the nitrogen bases once hydrogen bonds were disrupted. Thomas (1954) and Shack (1958) observed that the UV spectrum of native DNA in the presence of Mg^{++} was unchanged. Fishman et al. (1967) reported that the effect of Mg^{++} on denatured DNA is similar to that of the action of Hg^{++} or Cu^{++}.

c. Ethidium Chloride

Ethidium bromide (2,7-diamino, 9-phenyl-phenanthridium-10-ethyl bromide) is a trypanocidal dye displaying several biological properties. It inhibits DNA-dependent nucleic acid polymerases such as Escherichia coli nucleotidyl transferase (EC 2.7.7.7) in vitro (Elliott, 1963) and forms soluble metachromatic complexes with nucleic acids. The complexing of ethidium bromide with purine and pyrimidine nucleotides produces slight shifts to longer wavelengths of the dye. Purine compounds are more effective than pyrimidine in causing this shift. For binding of ethidium a polynucleotide must possess some form of base-paired secondary structure. Evidence has been presented that primary binding occurs by a process of intercalation between adjacent base-pairs, while secondary binding occurs by a "stacking" mechanism (Waring, 1966).

A marked increase in fluorescence of the dye occurs on binding (LePecq et al., 1964; LePecq and Paoletti, 1966). In the presence of

high salt concentration ethidium binds almost exclusively to double-stranded hydrogen-bonded polynucleotides. However, at low salt concentrations of 0.01 M and less two kinds of binding sites exist: (a) the same double-stranded site (primary site) as at high salt concentration (0.2 M NaCl, 0.2 M Tris-HCl, pH 7.5) which is characterized by an enhanced fluorescence and a certain spectral shift; and (b) a second binding site (secondary site) at which fluorescence efficiency is low but the absorption spectrum is the same as at the first kind of site (LePecq and Paoletti, 1967).

Binding of intercalative dyes causes a partial unwinding of the duplex structure in closed circular DNA (Gellert, 1967). Since the buoyant density of the DNA-dye complex is inversely related to the amount of dye bound, the buoyant density of the closed circular DNA-dye complex at saturation is greater than that of the linear or nicked circular-DNA dye complex. Thus, the dye may be used to detect and distinguish between the different tertiary structures of DNA (Radloff et al., 1967; Bauer and Vinograd, 1968).

5. Electron Microscopy

The visualization of DNA is accomplished through the use of the electron microscope. The original method of transforming a bulk DNA solution into a monomolecular layer consisted of spreading the molecules on an aqueous subphase (Kleinschmidt and Zahn, 1959). Certain polymers and many globular proteins in solution will produce insoluble surface films on flat aqueous solution due to surface denaturation of the protein (Chessman and Davies, 1954). The surface layer is considered to

exist as a molecular net of unfolded polypeptide chains (Kleinschmidt, 1968). The DNA (or RNA) is adsorbed to the polypeptide by the basic side groups of amino acid residues. This effectively brings the nucleic acids from a three-dimensional position in aqueous solution to a two-dimensional position (adsorbed to the polypeptide net). Cytochrome C is the protein of choice (Kleinschmidt, 1968; Haberman et al., 1967; Sarov and Becker, 1967). The adsorbed nucleic acid plus protein is then transferred to a solid support and dried. Electron micrographs are taken of preparations contrasted by depositing metal (at an angle of 5-10°) along the nucleic acid filaments (Kleinschmidt, 1968). Metals used are uranium (Kleinschmidt et al., 1963), platinum (Hall and Litt, 1958), platinum-carbon (McCrea and Lipman, 1967), platinum-palladium (Bode and MacHattie, 1968), and other metals and their oxides.

MATERIALS AND METHODS

A. Experimental Calves

The experimental calves were obtained at local livestock auctions and were of mixed breed and sexes and of different ages. Anaplasma marginale was propagated in splenectomized calves as previously described (Dimopoullos et al., 1960; Schrader and Dimopoullos, 1963; Dimopoullos and Bedell, 1964, 1965; and Gough and Dimopoullos, 1965).

B. Hematology

Blood was collected periodically and smears stained with Giemsa stain were examined by light microscopy. As soon as 50 to 80 percent of the erythrocytes were infected, the animal was exsanguinated and blood was collected in flasks with heparin sodium solution (0.3 ml containing 1000 U.S.P. units/ ml/50.0 ml of blood) as anticoagulant.

C. Isolation of DNA

Separation of erythrocytes from white blood cells and plasma was accomplished by centrifugation at 1085 x g for 20 minutes at 4°C. The erythrocytes were washed 3-5 times in 0.9% NaCl and diluted with an equal volume of 0.9% NaCl containing 0.1 M sodium citrate. The suspension was sonicated at 8 amperes for 90 seconds with a Branson Sonifier (model S75). Citrate was added to inhibit the action of nucleases. The sonicated preparation was then subjected to differential centrifugation as shown in Figure 1. Sediments were examined for the presence of marginal bodies as a guide to the course of further experimentation.

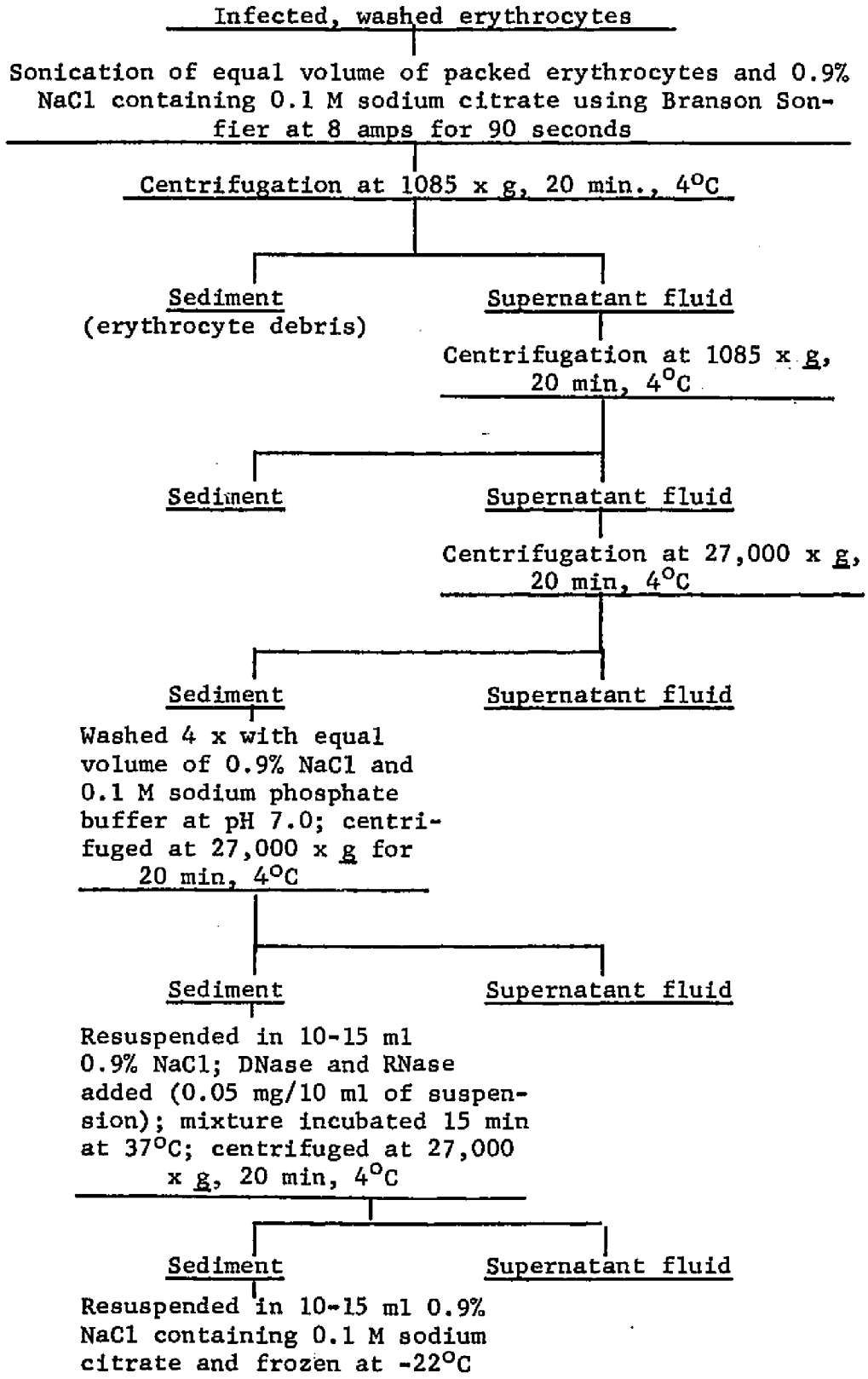

Figure 1. Sequence for the isolation of partially purified Anaplasma bodies.

Two methods were employed for the extraction of DNA (Marmur, 1961; Thomas et al., 1966) depending upon the experiments to be performed.

1. Method of Marmur (1961)

Frozen partially-purified marginal bodies were thawed in tap water. Approximately 2 grams of the sediment containing bodies were suspended in 25 ml of 0.15 M NaCl-0.1 M EDTA, pH 8.0. Lysis of bodies was induced by addition of 2.0 ml of 25% sodium lauryl sulfate solution (SLS) followed by 10 minutes incubation in a waterbath at 60°C. To the now viscous preparation, perchloric acid (final concentration 1 M) and an equal volume of a mixture of chloroform and isoamyl alcohol (24/1, v/v) were added. The flask was shaken for 30 minutes and the contents centrifuged at 1475 x g, for 5 minutes, 4°C. The upper aqueous layer was carefully transferred to a clean flask using a pipette whose narrow tip had been cut away and the edge fire-polished so as to reduce damage due to shear forces. Chloroform-isoamyl alcohol deproteinizations were repeated until the interfacial precipitate had all but disappeared, as described in Figure 2. Two volumes of 95% ethanol were layered carefully on top of the final aqueous layer precipitating the DNA which was wound on a glass rod and dissolved in 10-15 ml of 0.1 x SSC. The saline-citrate concentration was adjusted to that of 1 x SSC with 10 x SSC which was followed by incubation for 30 minutes at 37°C with RNase (50 μg/ml). The deproteinization steps were repeated with the DNA finally being dissolved in a volume of 9.0 ml of 0.1 x SSC. Finally, if the yield appeared sufficient, 1.0 ml of 3 M acetate - 1 x 10^{-3} M EDTA (pH 7.0) was added.

Partially purified marginal body suspension; 50 ml 0.15 M NaCl - 0.1 M EDTA, pH 8.0; centrifuged 15,900 x g, 20 min, 4°C; resuspended in 25 ml of same buffer; 2 ml of 25% SLS; incubate at 60°C for 10 min; perchloric acid to 1 M; equal vol. of chloroform-isoamyl alcohol 24-1 (v/v); shake, 30 min; centrifuged 1475 x g, 5 min, 4°C; repeated until little or no interfacial precipitate remained.

Chloroform layer

Aqueous layer

20 vols of 95% ethanol precipitated DNA; precipitate wound on glass rod; redissolved in 10-15 ml of 0.1 x SSC; made 1 x SSC with 10 x SSC; RNase for 30 min at 37°C.

Deproteinized using perchloric acid and chloroform-isoamyl alcohol; centrifuged 1475 x g, 5 min, 4°C; aqueous layer removed; 2 vol. 95% ethanol precipitated DNA; redissolved in 9.0 ml 0.1 x SSC; optional: added 1.0 Ml of 3 M acetate - 1 x 10^{-3} M EDTA (pH 7.0); DNA precipitated by 0.54 vol. isopropyl alcohol added dropwise; spooled on glass rod; dissolved in 1 x SSC (1 drop of chloroform added as preservative).

Figure 2. Isolation of DNA from Anaplasma marginal bodies by the method of Marmur (1961) with slight modifications.

Isopropyl alcohol (0.54 volume) was then added dropwise while spooling the DNA on a glass rod. DNA was stored dissolved in 1 x SSC along with a few drops of chloroform as preservative.

2. Phenolic Extraction (Thomas et al., 1966)

More often the infected erythrocytes were freed of white cells and plasma by aspirating off the buffy coat and suspending in 1 x SSC. DNA was extracted from the packed erythrocytes without any other pretreatments except those designed to inhibit or destroy nucleases.

The infected erythrocytes were incubated with both DNase and RNase (75 µg/ml) at 37°C for 15 minutes. After the cells were washed and resuspended in 1 x SSC, pronase (2 mg/ml) and SLS (2% final concentration) were added. The mixture was incubated for 7 hours at 37°C with occasional shaking. An equal volume of redistilled phenol saturated with 1 x SSC was added and the suspension rolled (60 rev/min) for 30 minutes. After chilling to 0°C the suspension was centrifuged at 1510 x g, and the phenol removed with a pipette. RNase (20 µg/ml) was added, and finally the solution was dialyzed against the buffer of choice, as described in Figure 3. This method of extraction was employed when DNA was to be tested with Mg^{++} and formaldehyde.

For the analytical centrifugations, T_M determination, and reaction with ethidium chloride, a combination of both the Marmur and phenolic extraction procedures was employed. Essentially, the phenol extraction method was followed up to the dialysis step. Instead of dialysis, perchloric acid (1 M final concentration) and chloroform-isoamyl alcohol

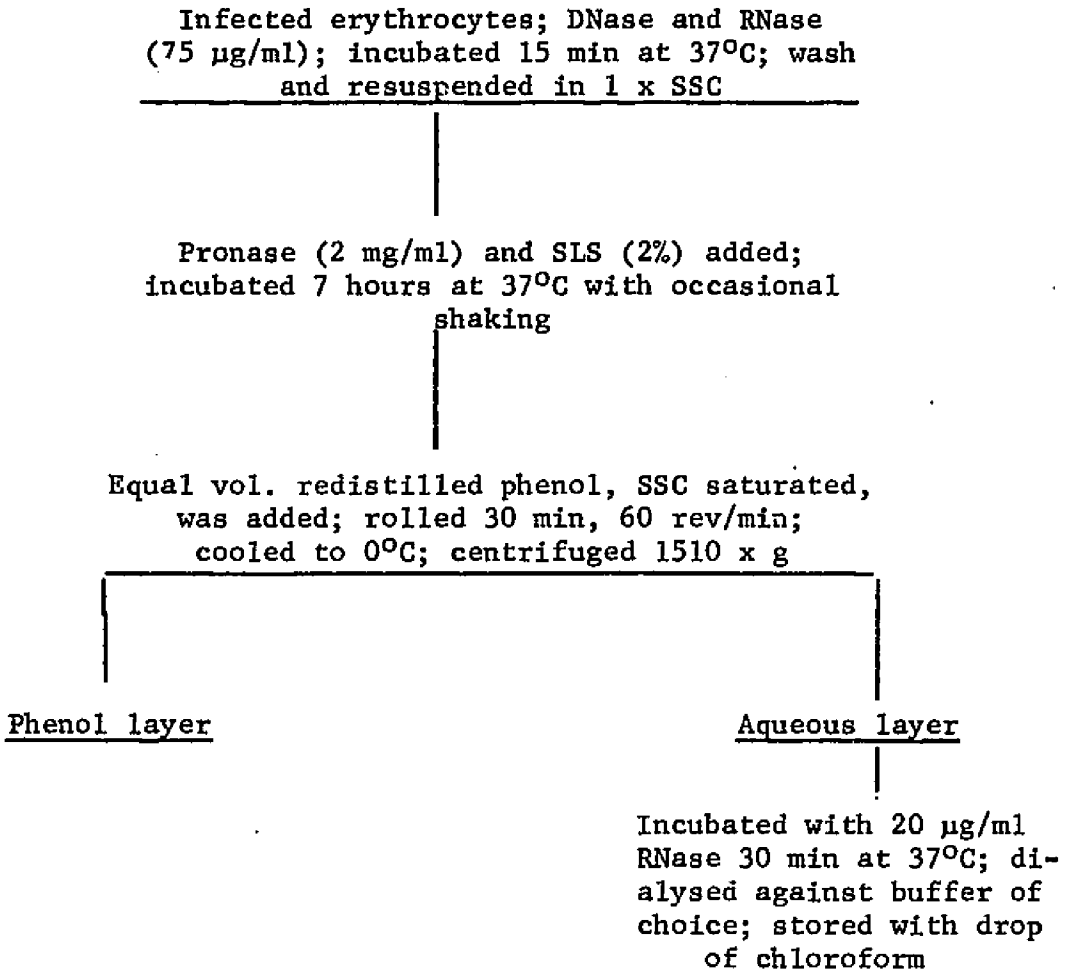

Figure 3. Scheme for phenolic extraction of DNA from A. marginale (Thomas et al., 1966).

deproteinizations were performed repeatedly. DNA was then precipitated with 2 volumes of 95% ethanol and spooled on a glass rod. The final step involved dissolving the DNA in the desired buffer.

D. Chemical Analysis

1. Hydrolysis

The DNA (extracted by Marmur's method) was dried in a vacuum oven at 40°C. A 0.5 mg sample of dried DNA was placed into a tube containing 0.5 ml of 88% formic acid. The tube was sealed with an oxygen-gas flame and hydrolysis was accomplished by placing the sealed tube in o-dichlorobenzene at 175°C for 30 minutes. After cooling, the tube was opened and the contents evaporated to dryness under a stream of nitrogen. The residue was dissolved in 25 µl of 1 N HCl and was chromatographed and quantitated as described below.

2. Descending Chromatography

Whatman No. 1 filter paper was cut to measure 20 x 50 cm. and divided into 7 lanes. Onto one lane 8 µl of the hydrolysate were spotted. Adenine (A), G, C, thymine (T), and uracil (U) served as controls. The solvent used to develop the chromatograms consisted of 12 N HCl-2-propanol-H_2O (16.7-65-18.3-v/v/v). Development times were approximately 20 hours, after which the chromatogram was removed and dried in an atmosphere of NH_4OH to neutralize the HCl.

3. Quantitation

Under long wave UV radiation the separated bases appear as dark spots against a background of general paper fluorescence. The spots and identical areas from blank lanes were cut out and each was shredded and

placed overnight in a test tube containing 5.0 ml of 0.1 N HCl for elution. The micromolar concentration of each base was determined spectrophotometrically in a Beckman DB Spectrophotometer employing the following molar extinction coefficients (Bendich, 1957):

	Wavelength at maximum absorption (nm)	E x 10^{-3}	$\frac{\mu \text{ moles}}{\text{OD unit}}$
Adenine	262.5	12.6	0.0794
Guanine	249	11.1	0.0901
Uracil	260	8.15	0.123
Thymine	265	7.95	0.126
Cytosine	283	10.0	0.100

The percentage of each base was calculated by dividing the number of micromoles of each by the total number of micromoles of all bases in the sample and multiplying by 100.

E. Thermal Denaturation

A measurable hyperchromicity at the absorption maximum (260 nm) accompanies the thermal denaturation of DNA. DNA in 1 x SSC was diluted to 20 µg/ml (an absorbance of about 0.400). The sample and reference (1 x SSC) in glass-stoppered cuvettes, were placed in a Beckman Model DU Spectrophotometer adapted for melting temperature determinations. The initial absorbance at 25°C was recorded and the temperature abruptly raised to 50°C. After degassing the sample, the temperature was further increased at the rate of about 1°C every 5 minutes. The absorbance,

corrected for the concentration dilution, divided by the initial absorbance at 25°C was plotted versus the temperature of the solution (Mandel and Marmur, 1968). The temperature corresponding to half the final increase in relative absorbance was designated the T_M. The T_M is dependent upon GC content and its determined value is influenced by the ionic strength of the solvent (Felsenfeld and Miles, 1967).

F. Chemical Reactions

1. Formaldehyde

The UV spectrum of DNA in 1 x SSC was taken in the absence of formaldehyde and just after the addition of formaldehyde (final concentration 2%). Samples were incubated for 5.5 hours at 37°C and again the UV spectrum was taken. Absorbance versus wavelength (nm) was plotted for each determination.

2. Magnesium Ion

Untreated DNA was dissolved in 5 mM NaCl and divided into two aliquots. One sample was placed in a waterbath at 100°C for 5 to 10 minutes and then chilled immediately by plunging it into an icebath for at least two minutes. A sample so treated was referred to as heated and fast-cooled DNA (HFC). To untreated (native) and HFC samples was added $MgCl_2$ to a final concentration of 0.04 M. The UV spectrum of the samples, using 5 mM NaCl with or without $MgCl_2$ as the reference solution was determined in a Beckman DB Spectrophotometer.

3. Ethidium Chloride

Ethidium chloride (10 µg/ml of 0.1 M NaCl-0.1 M Tris, pH 7.5) was added to samples of native DNA in concentrations ranging from 4 to 10 µg/ml. The dye was added to HFC DNA in differing concentrations. Relative fluorescence, recorded in an Aminco Bowman Spectrofluorometer was plotted versus DNA concentration (LePecq and Paoletti, 1968).

G. Electron Microscopy

Electron microscope grids (copper, 300 mesh) were coated with parlodion. Fresh slices of mica were shadowed with carbon and then the film was scored into grid-sized squares and floated onto the surface of glass-distilled water contained in a petri dish. The parlodion coated grids were touched to floating carbon squares and stored in another petri dish for future use. A third petri dish whose lip had been ground flat was lightly coated with paraffin (Sarov and Becker, 1967); McCrea and Lipman, 1967). This dish was filled with 0.25 M ammonium acetate (made with glass-distilled water) to a positive meniscus and the surface scraped clean with a stainless steel bar. A stainless steel ramp was placed in the ammonium acetate at approximately a 30° angle. The concentration of DNA in solution was estimated by assuming that 0.02 absorbance units corresponded to 1 µg DNA/ml. The concentration of DNA was adjusted to 5 µg/ml with 0.04% cytochrome C in 4 mM NaCl. One hundred microliters of this solution were slowly drawn into a microsyringe fitted with a length of plastic tubing. Droplets were slowly deposited on a stainless steel ramp so that after approximately 25 µl

had been deposited the drop would roll rapidly down the ramp onto the clean ammonium acetate subphase. The previously prepared grids were punched through the surface of the ammonium acetate, dipped 15-20 seconds in absolute ethanol, and blotted dry. The grids were shadowed in a vacuum evaporator, with 50 mg of platinum at an angle of 5-10° from two perpendicular directions.

All grids were observed in an RCA EMU-3 electron microscope. Electron micrographs were taken at a magnification of approximately 7×10^3 or higher.

H. Sedimentation Equilibrium Centrifugation

A stock solution of CsCl (optical grade powder, Harshaw Chemical Company) was prepared by dissolving 130 gm in 70 ml of 0.02 M Tris buffer, pH 8.5 (Mandel et al., 1968). The mixture to be centrifuged consisted of 0.84 ml of CsCl stock solution, 0.01 ml of bacteriophage SP8 DNA solution (about 50 µg/ml), and 0.04 ml of unknown DNA. The density was adjusted to 1.71 $g \cdot cm^{-3}$ ($\eta = 1.400$) with water or CsCl stock solution using the following relationship:

$$\rho\ 25^{\circ}C = 10.8601\ \eta_D 25^{\circ}C - 13.4974$$

Approximately 0.70 ml of the sample was placed in an ultracentrifuge cell with a 12 mm Kel-F centerpiece. The cell was seated in an An-D rotor and placed in a Beckman Model E Analytical Ultracentrifuge equipped with UV optics. The cell was centrifuged at 44,770 rpm at 25°C until equilibrium was attained (approximately 20 hours).

Buoyant density was calculated using the following equation (Sueoka, 1961):

$$P = Po + 4.2\omega^2 (r^2 - r_o^2) \times 10^{-10} \text{ g}\cdot\text{cm}^{-3}$$

The P_o was taken as 1.724 $g\cdot cm^{-3}$, while r and r_o was the distance from the center of rotation of the unknown and reference DNA peaks, respectively. The angular speed, ω, is expressed in radians/sec. For a speed of 44,770 rpm, $4.2\omega^2 \times 10^{-10}$ equals 0.0092.

The mole fraction GC of native DNA was calculated from the linear relation of Schildkraut et al. (1962):

$$GC = \frac{P - 1.660}{0.098}$$

Single-stranded DNA is heavier than its double-stranded counterpart by about 0.015 to 0.017 $g\cdot cm^{-3}$ (Schildkraut et al., 1962).

Photographs were taken and tracings of the film were made with a Spinco Analytrol Model R. The buoyant density of the DNA was calculated (as described above) from distances measured on the tracings.

RESULTS AND DISCUSSION

A. Introduction

Histochemical staining procedures have indicated the presence of both DNA and RNA in the Anaplasma body (Moulten and Christensen, 1955). Gough (1963) reported that DNA concentration reached a maximum in infected erythrocytes when marginal body counts were at their highest level. He also reported RNA concentration reached a peak three or four days after the maximum body count.

Ellender (1966) was able to isolate only DNA from partially purified marginal bodies. He reported that the mole percent for A, T, G, and C to be 32.4, 17.8, 34.3, and 15.5, respectively. It was concluded that it was single-stranded DNA, since he was unable to denature it with heat.

B. Isolation

No single method is suitable for isolation of DNA from all sources (Kit, 1963). The method of choice also depends on what is expected of the isolated DNA. Marmur's method (1961) is versatile, yielding DNA of good quality. The alcoholic precipitation perhaps causes degradation due to interfacial shearing forces. Phenol extraction (Thomas et al., 1966) eliminates the necessity for precipitation. Another advantage of this method is the use of pronase, which is added to inhibit the action of nucleases and aid in degradation of both cells and marginal bodies. A combination of both procedures was employed because this yielded the best preparations, i.e., gave the sharpest peaks in a CsCl gradient.

Chemical analysis was performed on DNA isolated from partially purified marginal bodies using the method of Marmur (1961). The method employing phenol was used when DNA was to be reacted with formaldehyde and Mg^{++}. For electron microscopy, isopycnic centrifugation, and ethidium chloride binding, DNA was isolated by a combination of both methods. However, it was found that the results were identical when either packed, infected erythrocytes or partially purified marginal bodies constituted the starting material.

C. Chemical Analysis

Hydrolysis of the purified DNA was accomplished by heating for 30 minutes in 88% formic acid at 175°C. R_f values on Whatman No. 1 filter paper for A, T, G, and C corresponded to the values obtained by Bendich (1957) and were 0.37, 0.90, 0.23, and 0.44, respectively. Figure 4 represents a chromatogram showing the relative positions of the standards and the bases in the hydrolyzate. The presence of U was not detected on the chromatograms.

The average base composition of Anaplasma DNA is shown in Table 1. Included in the Table are the values we obtained for E. coli and Anaplasma DNA, as well as those reported by Ellender (1966). E. coli DNA was used as a standard to check the technique. Differences in purity of starting preparations, excision, or elution procedures can account for the variations in these values and those of Ellender.

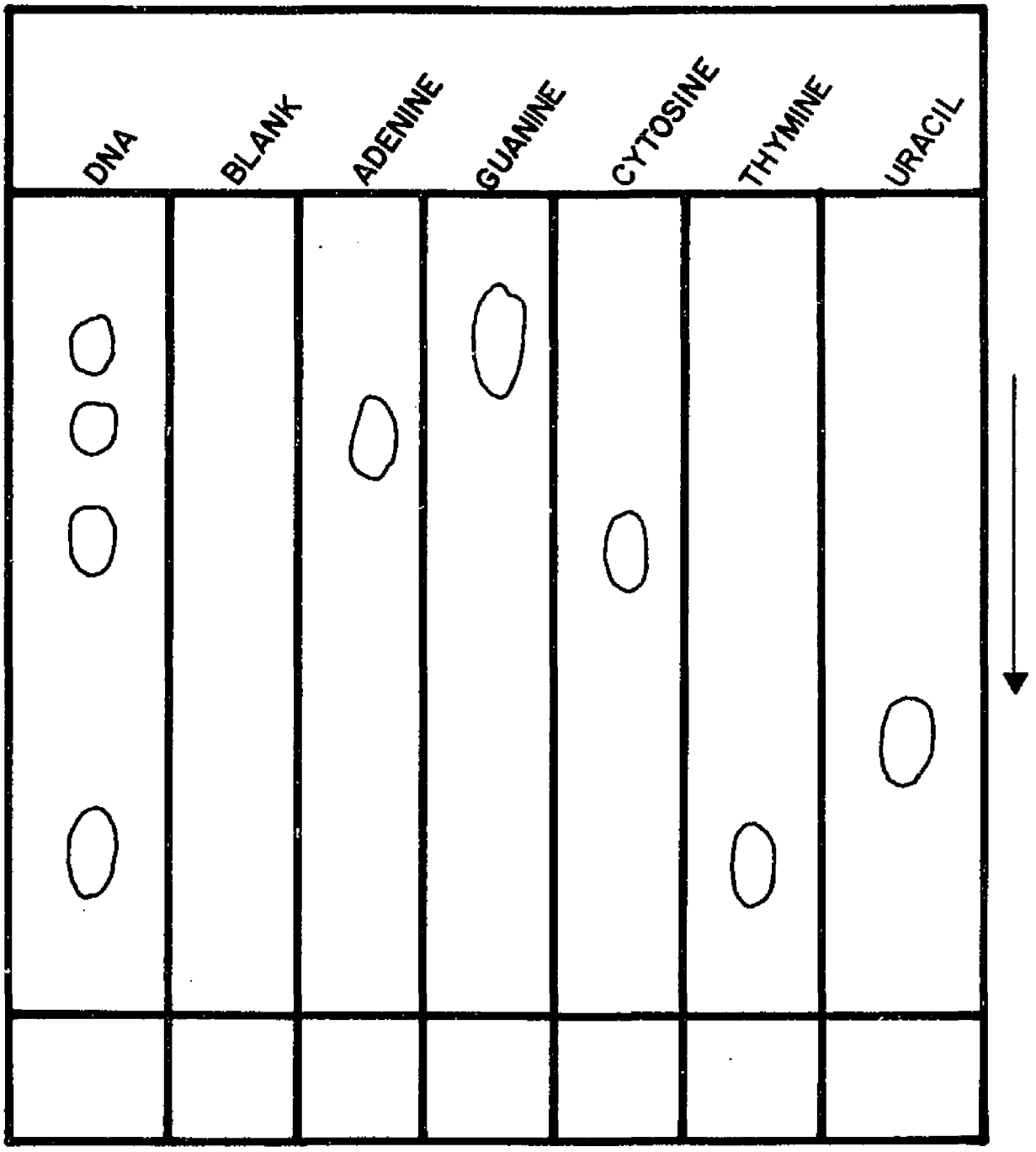

FIG.4. DIAGRAMMATIC REPRESENTATION OF A CHROMATOGRAM OF A HYDROLYSATE OF A. MARGINALE INFECTED ERYTHROCYTE DNA (DEVELOPED IN HCL-ISOPROPANOL-H_2O, 16.7/65/18.3-V/V/V).

Table I. Percent Base Composition of DNA Extracted by Marmur's Method (1961).

Organism	Relative percent base			
	Adenine	Guanine	Cytosine	Thymine
E. coli	25.6	26.4	24.4	23.6
A. marginale	33.8	34.5	20.2	11.5
A. marginale[a]	32.4	34.3	15.5	17.8

[a]Ellender, 1966.

D. Thermal Denaturation

Double-stranded, native DNA will exhibit a sharp increase in absorbance during heating in solvents of relative low ionic strength. Neither denatured (HFC) nor single-stranded DNA behaves in this fashion (Szybalski, 1967). Hyperchromicity occurs within a rather sharp temperature range and is dependent upon GC content. The presence of DNAs of different GC contents would therefore be detectable in this type of experiment.

The biphasic curve obtained with native DNA is shown in Figure 5. The T_M for the different phases was 84.9 and 92.0°C. Marmur's and Doty's (1962) relation between GC and T_M corresponds to that obtained by chemical analysis. The G/C and A/T ratios were 1.8 and 2.9, respectively

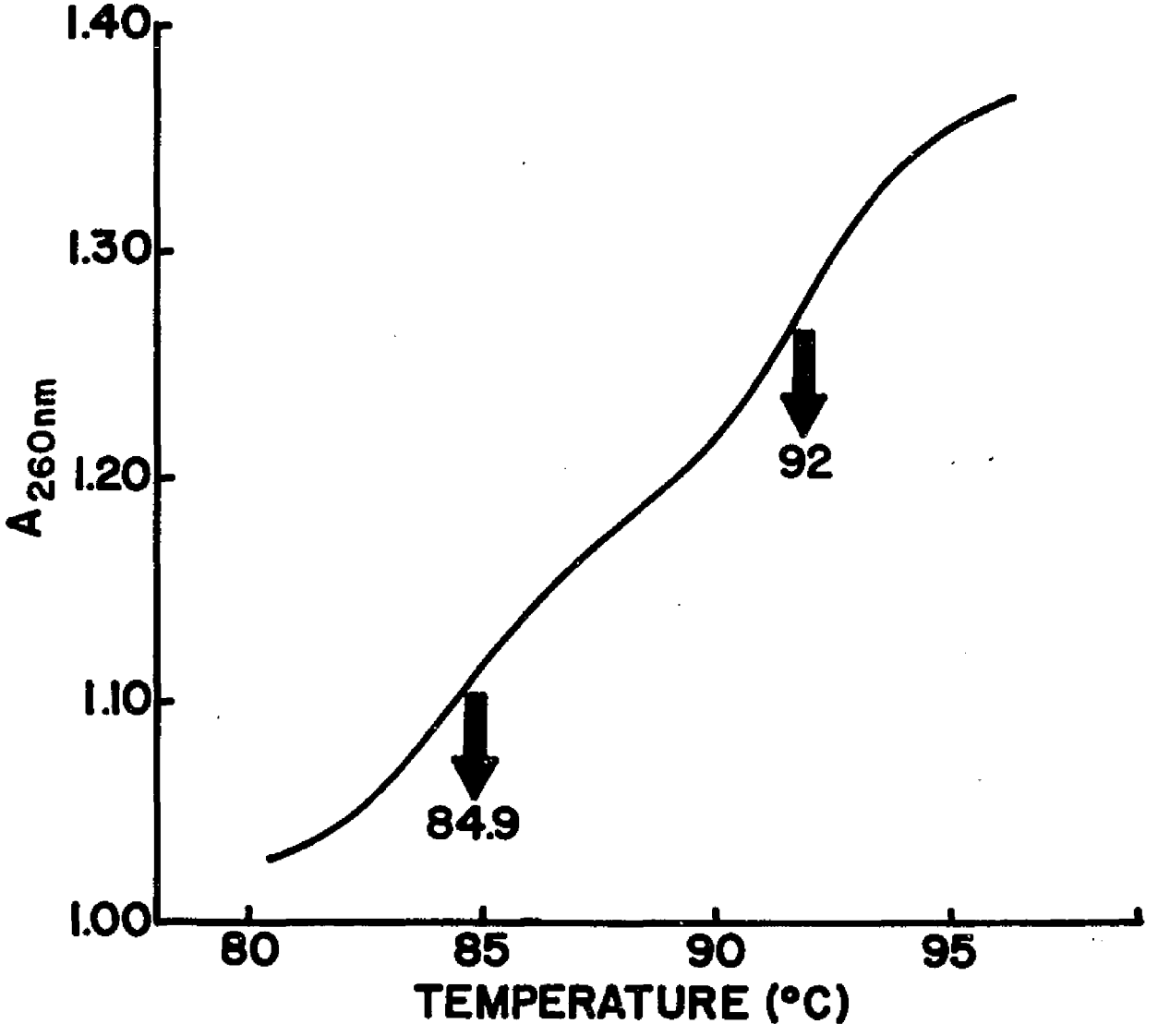

FIG.5. MELTING CURVE OF DNA FROM A. MARGINALE INFECTED ERYTHROCYTES.

(based on chemical analysis). This is characteristic of single-stranded DNA. Double-stranded DNA would yield ratios equal to 1.0. However, the hyperchromicity displayed by both species was characteristic of double-stranded DNA.

The DNA used for the base analysis was extracted by the method of Marmur (1961). For the other experiments DNA was extracted using a method employing pronase prior to lysis of the cells. One may postulate the presence of DNase in the enucleate erythrocyte. Therefore, one can say that Marmur's method allows for loss of at least part of the DNA molecule.

E. Chemical Reactions

1. Formaldehyde

The denaturing effect of formaldehyde on isolated DNA is well known. Figure 6 depicts the spectra of DNA samples in the presence and absence of formaldehyde. Incubation for 5.5 hours at 37°C in the presence of formaldehyde showed a difference in absorbance of about 4.8×10^{-2} absorbance units while the absence of formaldehyde between the zero and 5.5 hour control absorbance was 5×10^{-3} units. The former difference indicates denaturation while the latter is insignificant. Single-stranded DNA in the presence of formaldehyde regardless of the length of incubation would not have exhibited any significant increase in absorbance above that displayed immediately after addition of formaldehyde.

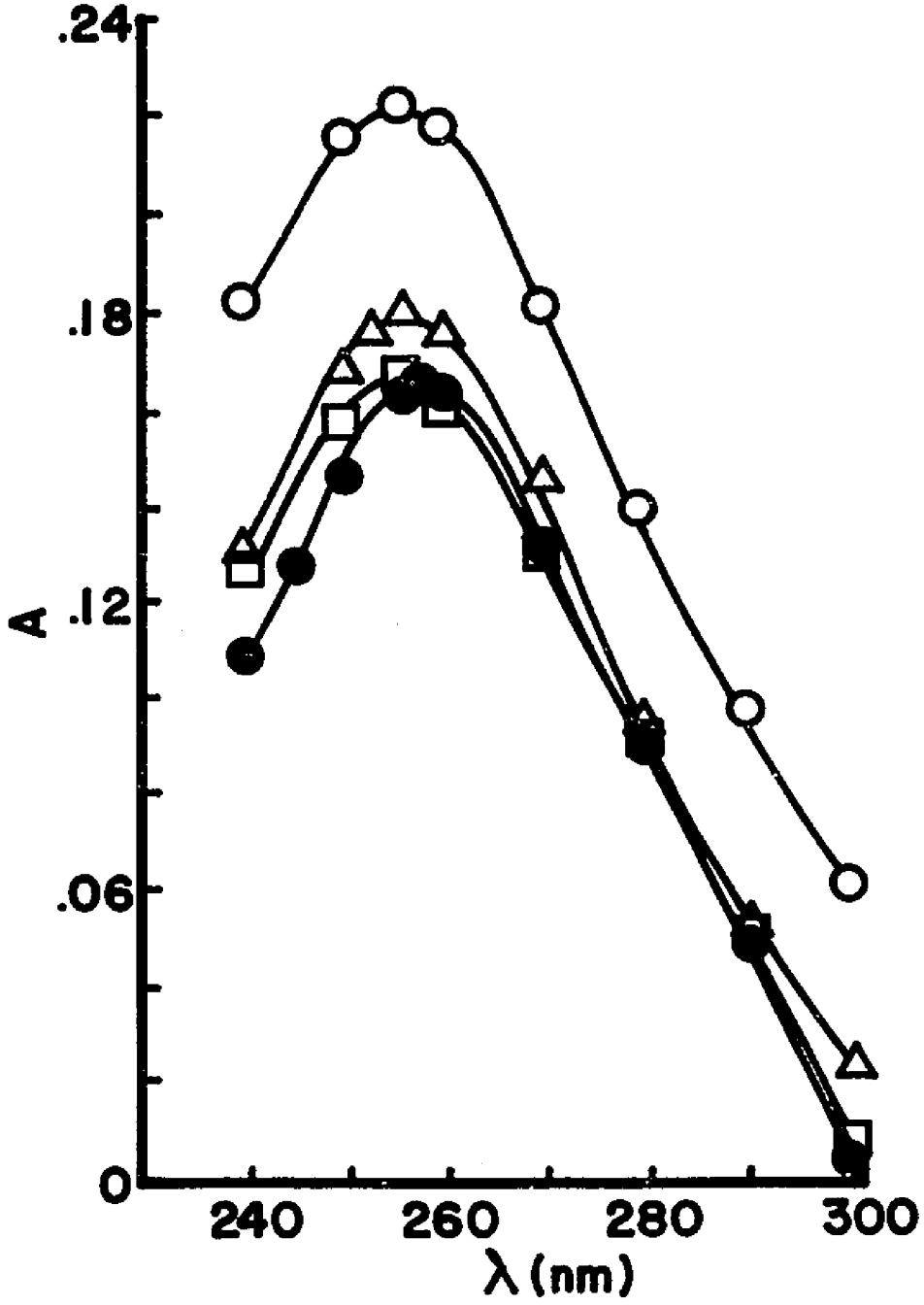

FIG. 6. DENATURING EFFECT OF FORMALDEHYDE WITH TIME. SYMBOLS: △, 0% HCHO (T=5.5 HRS.); ○, 2% HCHO (T=5.5 HRS.); ●, 0% HCHO (T=0); □, 2% HCHO (T=0).

2. Magnesium Ion

Native, double-stranded DNA has been shown to be unreactive to Mg^{++}. The DNA isolated from infected erythrocytes in the presence of 0.04 M Mg^{++} is unreactive, as shown in Figure 7. Denatured DNA, however, does react with Mg^{++} to shift its UV spectrum to higher wavelengths. Figure 8 clearly shows that the UV spectrum of HFC DNA is indeed shifted to higher wavelengths. This is interpreted to mean that the DNA is denaturable, and there, double-stranded in the native state.

3. Ethidium Chloride

The presence of secondary structure is a prerequisite for the binding of ethidium chloride to nucleic acid. Binding increases the fluorescence quantum efficiency of the dye. An increase in the amount of secondary organization (i.e., helical content) will proportionately increase the amount of dye that will bind. The difference in the slope of plots of relative fluorescence versus concentration of native and HFC DNA indicates loss of secondary organization attributable to the heating and fast-cooling procedure. This was shown to be the case, as seen in Figure 9. The only explanation for these results is that the isolated DNA was double-stranded.

F. Sedimentation Equilibrium Centrifugation

Centrifugation to equilibrium in a CsCl gradient provided a means for determining the buoyant density of DNA and the homogeneity of preparations. Figure 10 shows tracings made of a preparation of native DNA isolated from purified marginal bodies. The buoyant density of the two

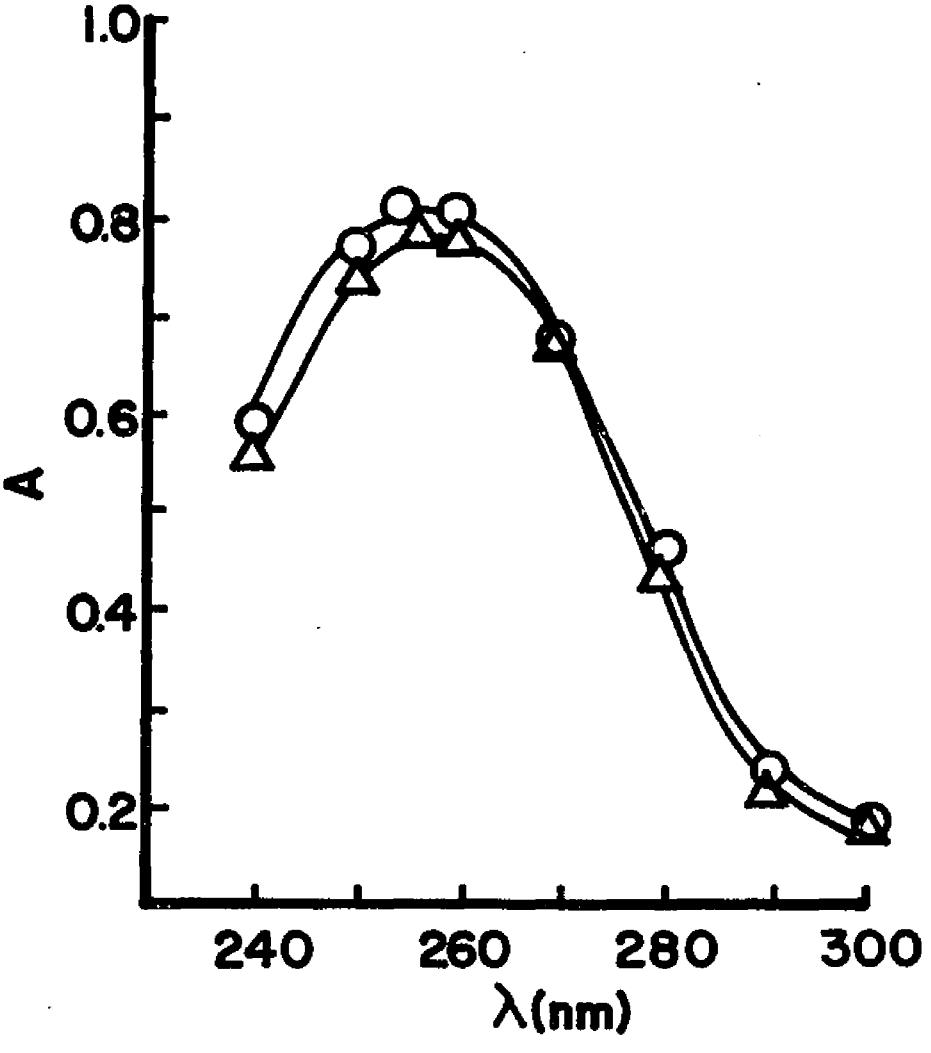

FIG.7. REACTION OF NATIVE DNA IN THE PRESENCE (O) AND ABSENCE (Δ) OF Mg^{2+}.

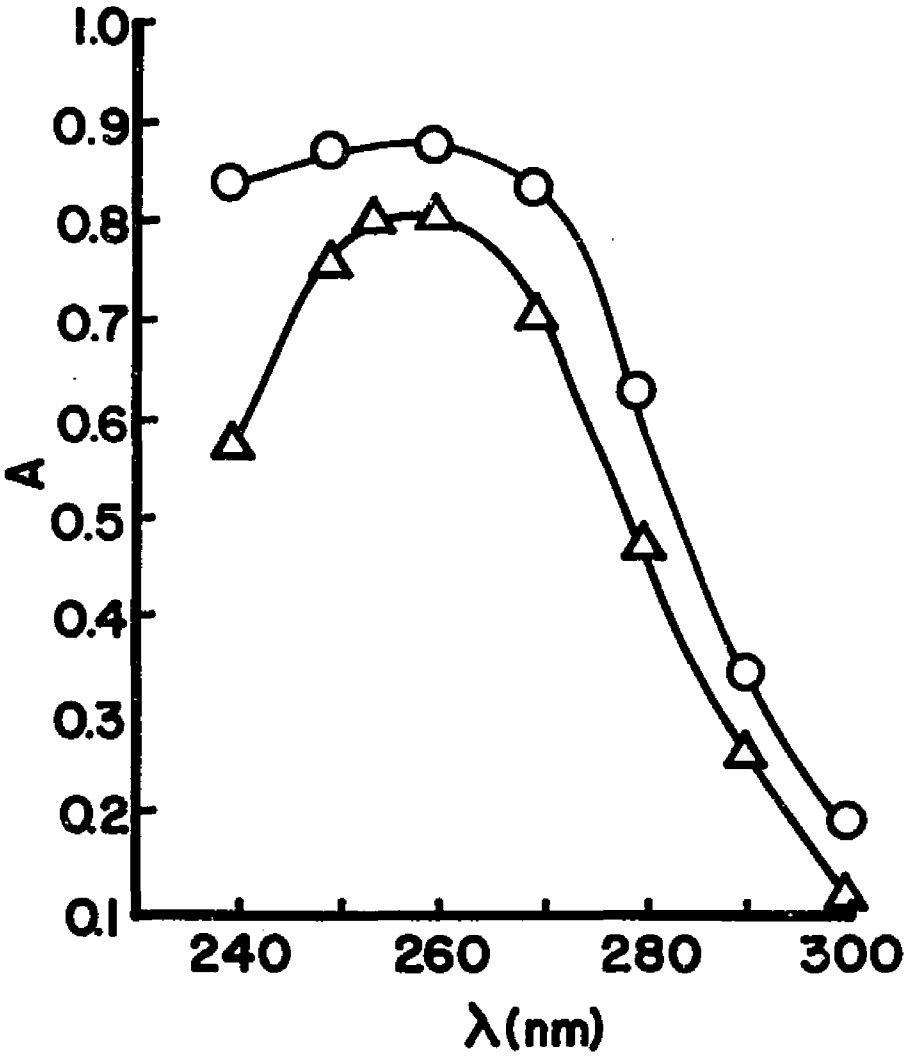

FIG.8. REACTION OF HFC DNA IN THE PRESENCE (O) AND ABSENCE (Δ) OF Mg^{2+}.

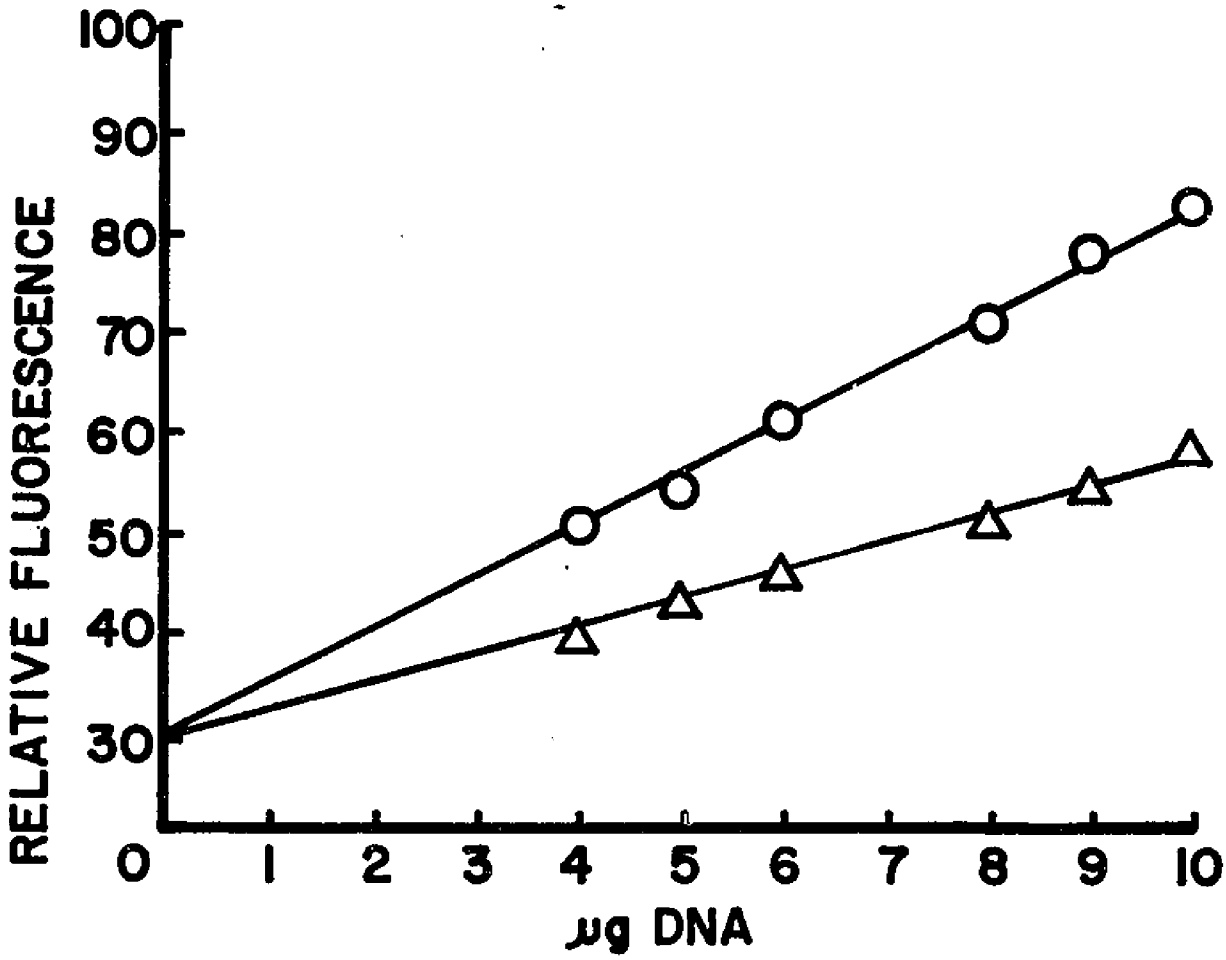

FIG.9. EFFECT OF ETHIDIUM CHLORIDE ON NATIVE (O) AND HFC (△) DNA.

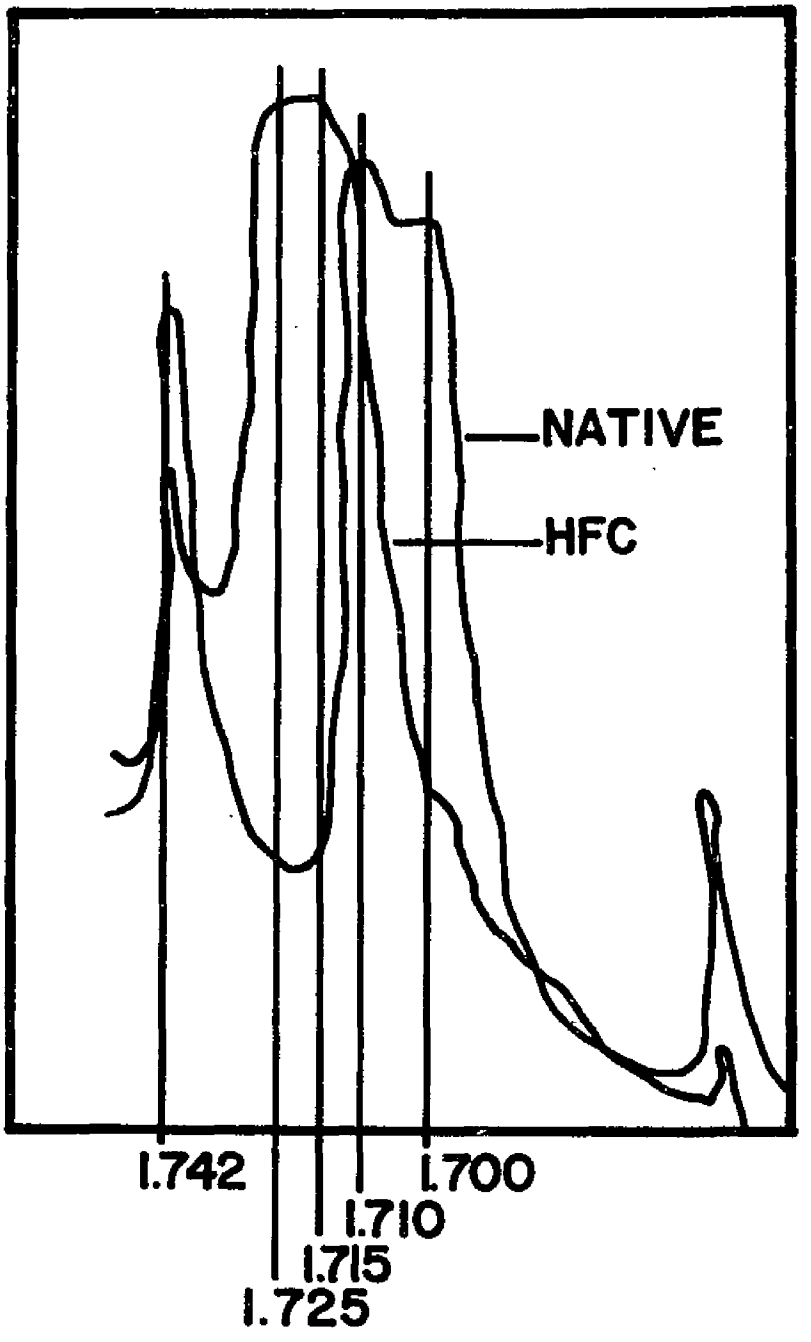

FIG. 10. DENSITOMETER TRACINGS OF ULTRAVIOLET PHOTOGRAPHS OF CsCl BUOYANT DENSITY GRADIENTS OF NATIVE AND HFC DNA. THE REFERENCE BAND TO THE LEFT IS NATIVE DNA OF BACTERIOPHAGE SP8.

peaks corresponded to 1.700 and 1.710 $g \cdot cm^{-3}$. The reference peak was taken as 1.742 $g \cdot cm^{-3}$ (for bacteriophage SP8 DNA). The HFC DNA displayed higher buoyant density, which is characteristic of double-stranded DNA.

To demonstrate which species of DNA was native to A. marginale the experiment shown in Figure 11 was performed. The first tracing, A, depicts DNA isolated from uninfected calf erythrocytes. No peak at 1.710 $g \cdot cm^{-3}$ was evident. Such a peak appeared as a trace in the sample from that calf at a time when 1% of its erythrocytes were infected. By the time the same calf was displaying a 58% infection the peak at 1.710 $g \cdot cm^{-3}$ was quite prominent, as it was in the 80% infected sample. The concentration of this DNA increased concomitantly with the amount of infection. The peak at 1.700 $g \cdot cm^{-3}$ corresponded to normal bovine DNA, while the 1.715 $g \cdot cm^{-3}$ peak was probably attributable to nuclear satellite DNA often observed in DNA preparations.

G. Electron Microscopy

Platinum-shadowed preparations were scanned for the presence of DNA molecules. Figure 12 is an electronmicrograph of DNA isolated from the erythrocytes of a calf infected with A. marginale. It appears to have had a circular conformation which had been broken. Unfortunately, this electronmicrograph was made before it was known that the preparations contained two species of DNA. One can only speculate as to whether this is an electron micrograph of bovine or A. marginale DNA. However, since bovine DNA has not been shown to be circular, it is highly suggestive that the DNA from A. marginale may indeed be circular.

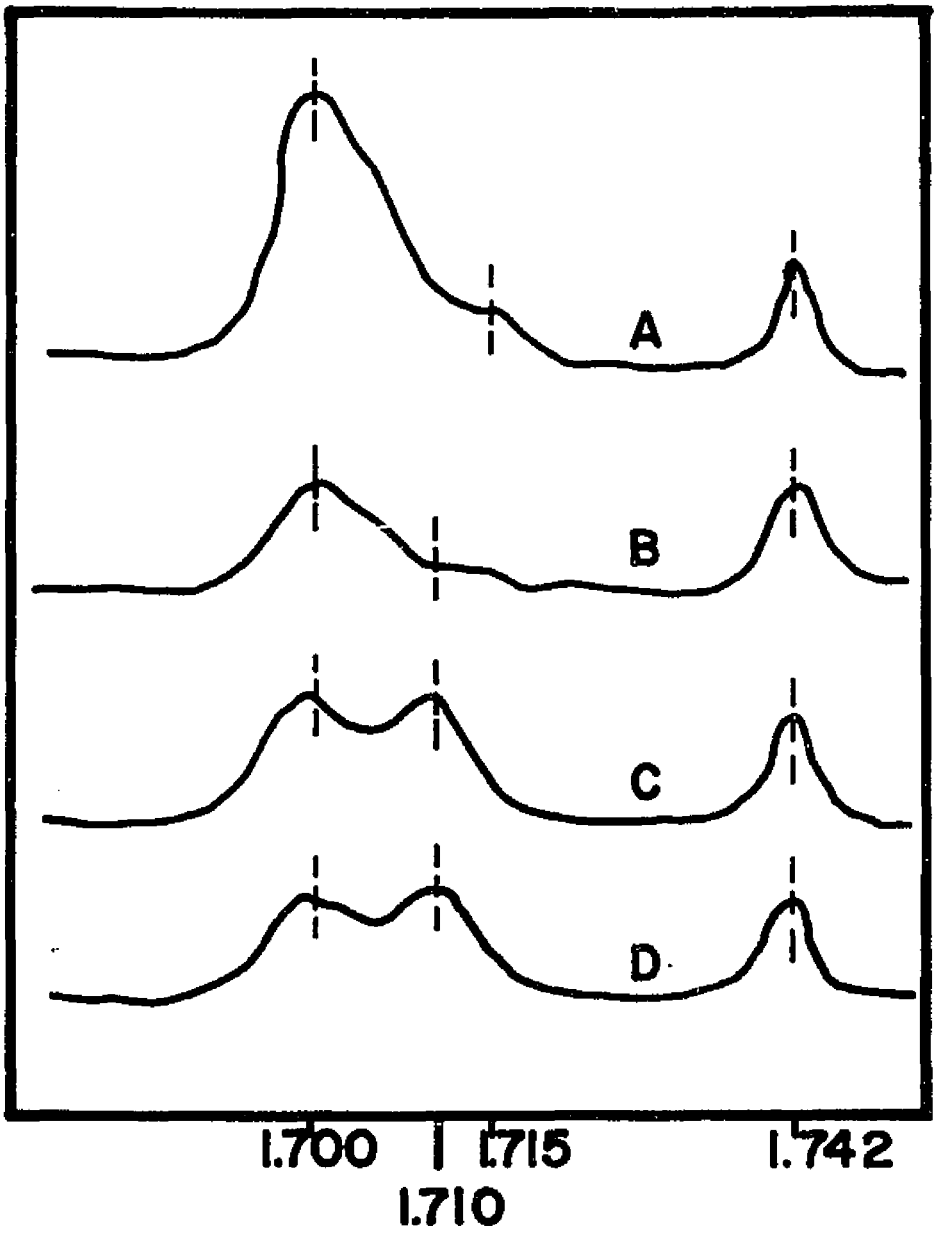

FIG. 11. PHOTOMULTIPLIER SCANNER TRACINGS AT 254nm OF DNA FROM LYSATES OF ERYTHROCYTES. A, NORMAL; B, 1% INFECTED; C, 58% INFECTED; D, 80% INFECTED. THE REFERENCE BAND TO THE RIGHT IS NATIVE DNA OF BACTERIOPHAGE SP8.

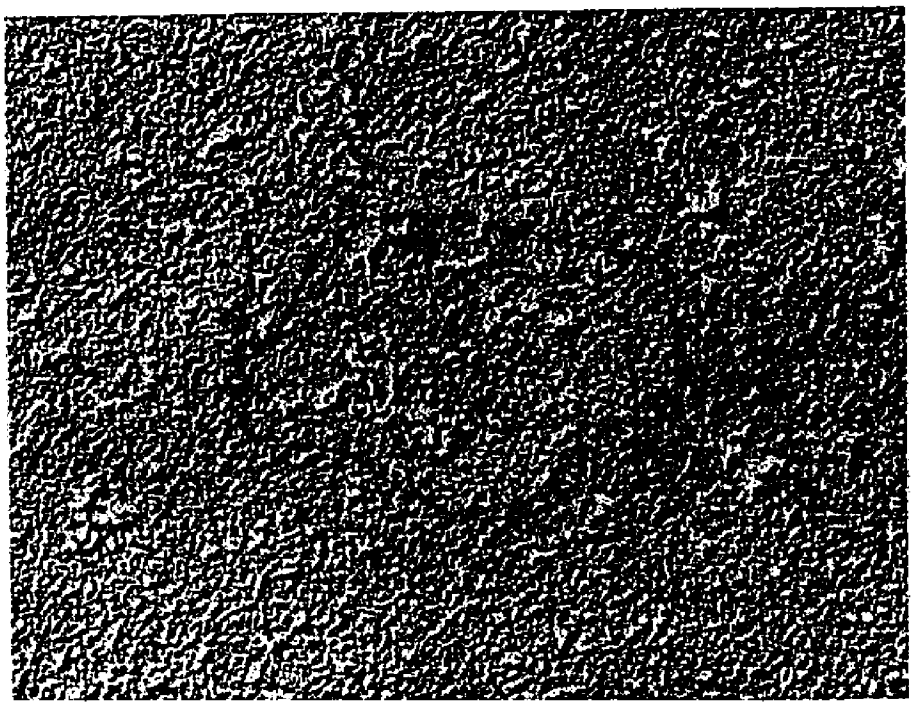

Figure 12. Electronmicrograph of DNA isolated from A. marginale infected erythrocytes.

SUMMARY AND CONCLUSION

This project was undertaken to characterize the DNA of Anaplasma marginale by physical and chemical means.

The methods of Marmur (1961) and Thomas et al. (1966) for extracing DNA, and the combination of both techniques proved successful.

Hydrolysis in 88% formic acid at 175°C for 30 minutes was deemed suitable for liberation of all bases. Chromatographic separation on Whatman No. 1 filter paper was accomplished using HCl-2-propanol-H_2O as the solvent. After running for about 20 hours (or about 40 cm.) the chromatograms were dried, the bases eluted, and quantitated. The mole percent G, C, A, and T. were 34.50, 20.18, 33.78, and 11.74, respectively, with molar ratios of A/T = 2.9 and G/C = 1.8. These data indicated single-stranded DNA.

A larger excess of purines to pyrimidines was found by the chromatographic method. Degradation of the nucleic acid by nucleases cannot be ruled out while employing Marmur's method (1961) of extraction. The erythrocyte must have attained the mature enucleate state through the action of nucleases. Therefore, one can postulate the preferential loss of pyrimidine-rich segments of the DNA molecule due to nuclease activity.

Reaction with formaldehyde, Mg^{++} and ethidium chloride were unmistakeably characteristic of double-stranded DNA. Further confirma- of that conclusion was obtained by the melting curves produced by heating in 1 x SSC. The T_M of A. marginale DNA was determined as 92°C, with a calculated mole %GC equal to 51.

Sedimentation equilibrium centrifugation in CsCl revealed the buoyant density of A. marginale DNA as equal to 1.710 $g \cdot cm^{-3}$, the density increasing to 1.725 $g \cdot cm^{-3}$ upon thermal denaturation.

Electron microscopy of isolated DNA revealed the presence of circular molecules in the preparations from infected erythrocytes. It was subsequently shown that experimental preparations contained two species of DNA. One species was demonstrated to be present only in infected erythrocytes. The other was thought to be DNA from leucocytes and reticulocytes which were present. No definite conclusions as to the origin of the DNA seen in Figure 12 can be made at this time.

In conclusion, the DNA from Anaplasma marginale has been shown to be a double-stranded molecule with a buoyant density equal to 1.710 $g \cdot cm^{-3}$ and a T_M of 92°C.

LITERATURE CITED

Alberts, B. M. 1967. Fractionation of nucleic acids by dextran - polyethylene glycol two-phase systems. In L. Grossman and K. Moldave (ed.), Methods in Enzymology, Vol. XIIa., pp. 566-581. Academic Press, Inc., New York.

Bauer, W. and J. Vinograd. 1968. The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV40 DNA in presence and absence of dye. J. Mol. Biol. 33:141-172.

Bendich, A. 1957. Methods for the characterization of nucleic acids by base composition. II. Determination of base composition by paper chromatography. In S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, Vol. III, pp. 715-723. Academic Press, Inc., New York.

Berns, K. I. and C. A. Thomas. 1961. A study of single polynucleotide chains derived from T2 and T4 bacteriophage. J. Mol. Biol. 3: 289-300.

Berns, K. I. and C. A. Thomas. 1965. Isolation of high molecular weight DNA from Hemophilus influenzae. J. Mol. Biol. 11:476-490.

Bode, V. C. and C. A. MacHattie. 1968. Electron microscopy of super helical circular λ DNA. J. Mol. Biol. 32:673-679.

Cane, R. H., T. E. Rogers, and G. T. Dimopoullos. 1963. The developmental cycle of Anaplasma marginale. Proc. 3rd Natl. Res. Conf. on Anaplasmosis, Manhattan, Kansas, pp. 19-22.

Carricaburu, J. B. 1957a. The clinical diagnosis of anaplasmosis. Proc. 3rd Natl. Res. Conf. on Anaplasmosis, Manhattan, Kansas, pp. 19-22.

Carricaburu, J. B. 1957b. The clinical aspects of anaplasmosis. Proc. 61st Ann. Meet. U. S. Livestock Sanit. Assoc., pp. 74-78.

Carter, C. E. 1950. Paper chromatography of purine and pyrimidine derivatives of yeast ribonucleic acid. J. Am. Chem. Soc. 72:1466-1471.

Chargaff, E. and H. F. Saidel. 1949. On the nucleoproteins of avian tubercle bacilli. J. Biol. Chem. 177:417-428.

Chargaff, E. and S. Zamenhof. 1948. The isolation of a highly polymerized desoxypentose-nucleic acid from yeast cells. J. Biol. Chem. 173:327-335.

Chessman, D. F. and J. T. Davies. 1954. Physiochemical and biological aspects of proteins at interfaces. Adv. Protein Chem. 9:439-501.

Christensen, J. F. 1956. Anaplasmosis. In Diseases of Cattle. Am. Vet. Publications, Evanston, Illinois, pp. 657-666.

Collins, C. J. and W. R. Guild. 1968. Irreversible effects of formaldehyde on DNA. Biochim. Biophys. Acta. 157:107-113.

Darlington, P. B. 1926. Anaplasmosis in cattle (galziete) found to exist in Kansas. N. Am. Vet. 7:39-41.

Darré, D. 1966. Lactate dehydrogenase studies in Anaplasmosis. I. Comparison between enzymatic activities of serum and erythrocytes from normal and infected calves. II. Evidence for lactate dehydrogenase activity in Anaplasma marginale. M. S. Thesis, Louisiana State University, Baton Rouge, Louisiana.

DeLey, J. 1967. The quick approximation of DNA base composition from absorbancy ratios. Antonie van Leeuwenhoek 33:203-208.

Descaseaux, J. 1924. L'Anaplasmose au Chili. Bull. Soc. Pathol. Exotrique 17:639-642.

Dikmans, G. 1933a. Anaplasmosis. V. The nature of Anaplasma. J. Am. Vet. Med. Assoc. 83:101-104.

Dikmans, G. 1933b. Anaplasmosis. VI. The morphology of anaplasmosis. J. Am. Vet. Med. Assoc. 83:203-213.

Dimopoullos, G. T. and D. M. Bedell. 1964. Flocculating properties of erythrocytic stromata: Observations on erythrocytes from normal deer and from calves infected with Eperythrozoon. Am. J. Vet. Res. 25:394-398.

Dimopoullos, G. T. and D. M. Bedell. 1965. Flocculating properties of erythrocytic stromata: Observations during convalescence from bovine anaplasmosis. Am. J. Vet. Res. 26:885-888.

Dimopoullos, G. T., G. T. Schrader, and L. E. Foote. 1960. Electrophoretic studies of bovine serum. III. Serum protein changes in anaplasmosis. Am. J. Vet. Res. 21:222-225.

DuBuy, H. G., C. F. T. Mattern, and F. L. Riley. 1965. Isolation and characteristics of DNA kinetoplasts of Leishmania enrietti. Science 147:754-756.

Eichhorn, G. L. 1962. Metal ions as stabilizers or destabilizers of the deoxyribonucleic acid structure. Nature 194:474-475.

Eichhorn, G. L. and P. Clark. 1965. Interactions of metal ions with polynucleotides and related compounds. V. The unwinding and rewinding of DNA strands under the influence of copper (II) ions. Proc. Natl. Acad. Sci. 53:586-593.

Eichhorn, G. L., P. Clark, and E. D. Becker. 1966. Interactions of metal ions with polynucleotides and related compounds. VII. The binding of copper (II) to nucleosides, nucleotides, and deoxyribonucleic acids. Biochemistry 5:245-253.

Ellender, R. D. 1966. Base composition of deoxyribonucleic acid isolated from Anaplasma marginale. M. S. Thesis, Louisiana State University, Baton Rouge, Louisiana.

Ellender, R. D. and G. T. Dimopoullos. 1967. Base composition and thermal denaturation of DNA isolated from Anaplasma marginale. Proc. Soc. Expt. Biol. and Med. 125:82-85.

Elliott, W. H. 1963. Effects of antimicrobial agents on deoxyribonucleic acid polymerase. Biochem. J. 86:562-567.

Espana, C., E. M. Espana, and D. Gonzales. 1959. Anaplasma marginale. I. Studies with phase contrast and electron microscopy. Am. J. Vet. Res. 20:795-805.

Felsenfeld, G. and H. T. Miles. 1967. The physical and chemical properties of nucleic acids. Ann. Rev. Biochem. 36:407-448.

Fishman, M. M., J. Isaac, S. Schwartz, and S. Stein. 1967. The binding of magnesium ion to DNA. Biochem. Biophys. Res. Comm. 29: 378-381.

Foote, L. E. 1954. New information on anaplasmosis. N. Am. Vet. 35: 19-21.

Foote, L. E., J. C. Geer, and Y. E. Stich. 1958. Electron microscopy of the Anaplasma body: Ultra-thin sections of bovine erythrocytes. Science 128:147-148.

Franklin, T. E. and H. E. Redmond. 1958. Observations on the morphology of Anaplasma marginale with reference to projections or tails. Am. J. Vet. Res. 19:251-253.

Gainer, J. H. 1961. Demonstration of Anaplasma marginale with acridine orange. Comparisons with the complement-fixation test and Wright's stain. Am. J. Vet. Res. 22:882-886.

Garon, C. F. 1966. Cation permeability and adenosine triphosphatase activity of the erythrocytic membrane in anaplasmosis. M. S. Thesis, Louisiana State University, Baton Rogge, Louisiana.

Gellert, M. 1967. Formation of covalent circles of lambda DNA by E. coli extracts. Proc. Natl. Acad. Sci. 57:148-155.

Gough, B. J. 1963. Studies of the changes in nucleic acid of blood in bovine anaplasmosis. M. S. Thesis, Louisiana State University, Baton Rouge, Louisiana.

Gough, B. J. and G. T. Dimopoullos. 1965. Biologic properties and characteristics of Anaplasma marginale: Effects of ionizing radiation on infectivity of whole blood preparations. Am. J. Vet. Res. 26:246-249.

Haberman, S., J. M. Hall, and B. A. Sanford. 1967. Isolation of human DNA and its demonstration by electron microscopy. Nature 215: 278-279.

Hall, C. E. and M. Litt. 1958. Morphological features of DNA macromolecules as seen with the electron microscope. J. Biophys. Biochem. Cytol. 4:1-4.

Haselkorn, R. and R. Doty. 1961. The reaction of formaldehyde with polynucleotides. J. Biol. Chem. 236:2738-2745.

Heppel, L. A. 1967. Techniques for the elution of nucleic acid components from paper. In: L. Grossman and K. Moldave (ed.), Methods in Enzymology. Vol. XIIa., pp. 316-317. Academic Press, Inc., New York.

Hiai, S. 1965. Effects of cupric ions on the thermal denaturation of nucleic acid. J. Mol. Biol. 11:672-691.

Hoard, D. E. and W. Goad. 1968. Products in the initial stages of digestion of polydeoxynucleotides by Pancreatic Deoxyribonuclease (DNase I). J. Mol. Biol. 31:585-606.

Hotchkiss, R. D. 1957. Isolation of sodium deoxyribonucleate in biologically active form from bacteria. In: S. P. Colowick and N. O. Kaplan (ed.), Methods in Enzymology, Vol. III, pp. 692-696. Academic Press, Inc., New York.

Kay, E. R. M. and A. L. Dounce. 1953. The preparation of sodium ribnucleate with the use of sodium dodecylsulfate. J. Am. Chem. Soc. 75:4041-4044.

Keck, K. and U. Hagen. 1964. Trennung von DNA- Baustrinen auf Celluloseschichten. Biochem. Biophys. Acta 87:685-687.

Kit, S. 1963. Deoxyribonucleic acids. In E. E. Allen and G. Machinnery (ed.), Ann. Rev. Biochem. Vol. 32, pp. 43-82. Annual Reviews, Inc., Palo Alto, California.

Kleinschmidt, A. K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. In L. Grossman and K. Moldave (ed.), Methods in Enzymology, Vol. XIIb., pp. 361-377. Academic Press, Inc., New York.

Kleinschmidt, A. K., A. Burton, and R. K. Sinsheimer. 1963. Electron microscopy of the replicative form of the DNA of the bacteriophage, ϕX 174, Science 142:961.

Kleinschmidt, A. K. and R. K. Zahn. 1959. Über desoxyribonucleinsäuremolekeln in protein-mischfilmen. Z. Naturforschung, 146:770-779.

Knittel, M. D., C. H. Black, W. E. Sandine, and D. K. Fraser. 1968. Use of normal probability paper in determining thermal melting values of deoxyribonucleic acid. Cand. J. Microbiol. 14:239-245.

Kream, J. and E. Chargaff. 1952. Procedures for the study of purine and pyrimidine deaminases in small amounts. J. Am. Chem. Soc. 74:4274-4277.

Laskowski, M. 1966. Pancreatic deoxyribonuclease I. In G. L. Cantoni and D. R. Davies (ed.), Procedures in Nucleic Acid Research, pp. 203-211. Harper and Row, New York.

Laskowski, M., Sr. 1967. Paper chromatography and characterization of oligodeoxyribonucleotides. In L. Grossman and K. Moldave (ed.), Methods in Enzymology, Vol. XIIa., pp. 281-304. Academic Press, Inc., New York.

Lehman, I. R. 1966. Exonuclease I (Phosphodiesterase) from Escherichia coli. In G. L. Cantoni and D. R. Davies (ed.), Procedures in Nucleic Acid Research, pp. 203-311, Harper and Row, New York.

LePecq, J.-B., P. Jeanteur, R. Emanoil-Raviocovitch, and C. Paoletti. 1966. A spectrofluorometric method for the study of transcription of single-stranded DNA by RNA polymerase. Biochim. Biophys. Acta 119:442-444.

LePecq, J. -B. and C. Paoletti. 1967. A fluorescent complex between ethidium bromide and nucleic acids. Physical-chemical characterization. J. Mol. Biol. 27:87-106.

Lewin, S. 1964. Some aspects of variations and errors in nucleic acid investigations. II. Limitations of preparative procedures, storage, and standardization techniques. Lab. Prac. 13:315-321.

Lewin, S. 1966. Reaction of Salmon Sperm deoxyribonucleic acid with formaldehyde. Arch. Biochem. and Biophys. 113:584-602.

Lotze, J. C. 1946. Further observations on the nature of Anaplasma. Proc. Helminthol. Soc. of Wash., D. C., 13:56-57.

Lotze, J. C. and M. J. Yiengst. 1942. Studies on the nature of Anaplasma. Am. J. Vet. Res. 3:312-320.

Mandel, M. and J. Marmur. 1968. Use of ultraviolet absorbance-temperature profile for determining the Guanine plus Cytosine content of DNA. In: L. Grossman and K. Moldave (ed.), Methods in Enzymology, Vol. XIIb., pp. 195-206. Academic Press, Inc., New York.

Mandel, M., C. L. Schildkraut, and J. Marmur. 1968. Use of CsCl Density Gradient Analysis for determining the Guanine plus Cytosine content of DNA. In L. Grossmann and K. Moldave (ed.), Methods in Enzymology, Vol. XIIb., pp. 184-195. Academic Press, Inc., New York.

Marigold, H. K. 1965. Nucleic acids and nucleotides. In E. Stahl (ed.), Thin Layer Chromatography. A Laboratory Handbook, pp. 440-460. Academic Press, Inc., New York.

Markham, R. and J. D. Smith. 1949. Chromatographic studies on nucleic acids. I. A technique for the identification and estimation of purines and pyrimidine bases, nucleosides, and related substances. Biochem. J. 45:294-298.

Marmur, J. 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. 3:208-218.

Marmur, J. and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.

Massie, H. R. and B. H. Zimm. 1965. The use of hot phenol in preparing DNA. Proc. Natl. Acad. Sci. 54:1641-1643.

McCrea, J. F. and M. B. Lipman. 1967. Strand - length measurements of normal and 5-iodo-2'-deoxyuridine-treated Vaccinia virus deoxyribonucleic acid released by the Kleinschmidt method. J. Virology 1:1037-1044.

Meyer, K. F. 1913. Die perniziöse Anämie der Rinder (Anaplasmosis). Handbuch der Pathogenen Microorganismen 7:531-539.

Moulton, J. E. and J. F. Christensen. 1955. The histochemical nature of Anaplasma marginale. Am. J. Vet. Res. 16:377-380.

Oglesby, W. T. 1962. Financial losses from anaplasmosis. Proc. 4th Natl. Anaplasmosis Conf., Reno, Nevada, pp. 1-2.

Osawa, S. and A. Sibatani. 1967. Chromatographic separation of RNA from DNA on MAK columns. In L. Grossman and K. Moldave (ed.), Methods in Enzymology, Vol. XIIa., pp. 678-684. Academic Press, Inc., New York.

Penha, A. M. 1930. Comportement des anaplasmes et des corpuscles de Jolly viv-avis de la reaction nucleaire de Feulgen. Comp. Rend. Soc. Biol. 103:1331-1332.

Peterman, M. L. and R. M. Lamb. 1948. The nucleohistone of beef spleen. J. Biol. Chem. 176:685-693.

Philip, C. B. 1956. Comments on the classification of the order Rickettsiales. Can. J. Microbiol. 2:261-266.

Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. 57:1514-1521.

Randerath, K. 1966. Nucleic acid constituents and nucleotide coenzymes. In Thin Layer Chromatography, 2nd ed., pp. 219-234. Academic Press, Inc., New York.

Rhaese, H. J. and E. Freeze. 1968. Chemical analysis of DNA alterations. I. Base liberation and backbone breakage of DNA and oligodeoxyadenylic acid produced by hydrogen peroxide and hydroxylamine. Biochim. Biophys. Acta 155:476-490.

Rhaese, H. J., E. Freese, and M. S. Melzer. 1968. Chemical analysis of DNA alterations. II. Alteration and liberation of bases of deoxynucleotides and deoxynucleosides induced by hydrogen peroxide and hydroxylamine. Biochim. Biophys. Acta 155:491-504.

Ristic, M. 1960. Anaplasmosis. In Advances in Veterinary Science, Vol. 6, pp. 111-192. Academic Press, Inc., New York.

Ristic, M. 1965. Piroplasms. In Progress in Protozoology, Excerpta Medica Found., New York, pp. 32-33.

deRobertis, E. and B. Epstein. 1951. Study of anaplasmosis in bovine red blood cells. Proc. Soc. Exptl. Biol. and Med. 77:254-258.

Roby, T. O. 1960. A review of the biological nature of Anaplasma marginale. Proc. 58th Ann. Meet. U. S. Livestock Sanit. Assoc., pp. 88-94.

Sarov, I. and Y. Becker. 1967. Studies of vaccinia virus DNA. Virology 33:369-375.

Schildkraut, C. L., M. Mandel, S. Levisohn, J. E. Smith-Sonneborn, and J. Marmur. 1962. Deoxyribonucleic acid base composition and taxonomy of some protozoa. Nature 196:795-796.

Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430-443.

Schrader, G. T. and G. T. Dimopoullos. 1963. Studies of bovine erythrocytes in anaplasmosis. III. Partition of erythrocytic phospholipids. Am. J. Vet. Res. 24:283-286.

Seibert, F. B. 1940. Removal of the impurities in nucleic acid and polysaccharide from tuberculin protein. J. Biol. Chem. 133: 593-604.

Sevag, M. G., D. D. Lackman, and J. Smolens. 1938. The isolation of the components of Streptococcal nucleo-proteins in serologically active form. J. Biol. Chem. 124:425-436.

Shack, J. 1958. On the recognition and estimation of denatured deoxyribonucleate. J. Biol. Chem. 233:677-680.

Simpson, C. F. 1967. Morphologic and histochemical nature of Anaplasma marginale. Amer. J. Vet. Res. 28:1055-1065.

Smith, T. and F. L. Kilborne. 1893. Investigations into the nature, causation, and prevention of Texas or southern cattle fever. U. S. Dept. Ag., Bureau Animal Indust. Bull. 1:1-301.

Smith, J. D. and M. G. P. Stoker. 1951. The nucleic acids of Rickettsia burneti. Brit. J. Exptl. Pathol. 32:433-441.

Soehner, R. L., G. A. Gentry, C. C. Randall. 1965. Some physicochemical characteristics of equine abortion virus nucleic acid. Virology 26:394-405.

Staehelin, M. 1958. Reaction of tobacco mosaic virus nucleic acid with formaldehyde. Biochim. Biophys. Acta 29:410-417.

Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390.

Sueoka, N. 1961. Variation and heterogeneity of base composition of deoxyribonucleic acids: a compilation of old and new data. J. Mol. Biol. 3:31-41.

Szybalski, W. 1967. Effects of elevated temperatures on DNA and some polynucleotides, denaturation, renaturation, and cleavage of glycosidic and phosphate ester bonds. In A. H. Rose (ed.), Thermobiology, pp. 73-122. Academic Press, Inc., London.

Theiler, A. 1910. Anaplasma marginale (genus nov. et Species nov.) Un nouveau protozaire du betail. Bull. Soc. Pathol. Extique 003: 135-137.

Thomas, C. A. 1954. The interaction of $HgCl_2$ with sodium thymonucleate. J. Amer. Chem. Soc. 76:6032-6034.

Thomas, C. A., K. I. Berns, and T. J. Kelley. 1966. Isolation of high molecular weight DNA from bacteria and cell nuclei. In G. L. Cantoni and D. R. Davies (ed.), Procedures in Nucleic Acid Research, pp. 535-540. Harper and Row, New York.

Vischer, E. and E. Chargaff. 1948a. Composition of the pentose nucleic acids of yeast and pancreas. J. Biol. Chem. 176:715-734.

Vischer, E. and E. Chargaff. 1948b. The separation and quantitative estimation of purines and pyrimidines in minute amounts. J. Biol. Chem. 176:703-714.

Wallace, W. R. and G. T. Dimopoullos. 1965. Catalase activity in Anaplasma marginale. J. Bacteriol. 90:309-311.

Waring, M. J. 1966. Structural requirements for the binding of ethidium to nucleic acids. Biochim et Biophys. Acta 114:234-244.

Wheeler, G. P. and J. A. Alexander. 1957. Studies with mustards. Effects of N_2 mustard treatment of DNase and DNA upon the enzymic degradation of DNA. Biochem. and Biophys. 72:476-484.

Whitfeld, P. R. 1953. Studies on the nucleic acids of the malarial parasite, Plasmodium berghei. Aust. J. Biol. Sci. 6:234-243.

Wyatt, G. R. 1951. The purine and pyrimidine composition of deoxypentose nucleic acids. Biochem. J. 48:584-590.

Wyatt, G. R. and S. S. Cohen. 1953. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethylcytosine. Biochem. J. 55:774-782.

Zamenhof, S., H. E. Alexander, and G. Leidy. 1953. Studies on the chemistry of the transforming activity. I. Resistance to physical and chemical agents. J. Exptl. Med. 98:373-397.

Zamenhof, S. and E. Chargaff. 1951. Separation of deoxypentose and pentose nucleic acids. Nature 168:604-605.

Zamenhof, S., G. Leidy, H. E. Alexander, P. L. FitzGerald, and E. Chargaff. 1952. Purification of the desoxypentose nucleic acid of Hemophilus influenzae having transforming activity. Arch. Biochem. and Biophys. 40:50-55.

Zittle, C. A. 1945. Ribonuclease. II. Mononucleotides in commercial ribonucleic acids and their effect on ribonucleinase. J. Biol. Chem. 160:527-533.

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EXAMINATION AND THESIS REPORT

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Major Field: Microbiology

Title of Thesis: Characterization of the DNA from Bovine Erythrocytes Infected with Anaplasma marginale.

Approved:

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Date of Examination:

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